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Competitive Binding of Bismuth to Transferrin and Albumin in Aqueous Solution and in Blood Plasma*

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Several bismuth compounds are currently used as antiulcer drugs, but their mechanism of action is not well established. Proteins are thought to be target sites. In this work we establish that the competitive binding of Bi^{3+} to the blood serum proteins albumin and transferrin, as isolated proteins and in blood plasma, can be monitored via observation of ^{1}H and ^{13}C NMR resonances of isotopically labeled [ϵ-^{13}C]Met transferrin. We show that Met^{132} in the I132M recombinant N-lobe transferrin mutant is a sensitive indicator of N-lobe metal binding. Bi^{3+} binds to the specific Fe^{3+} sites of transferrin and the observed shifts of Met resonances suggest that Bi^{3+} induces similar conformational changes in the N-lobe of transferrin in aqueous solution and plasma. Bi^{3+} binding to albumin is nonspecific and Cys^{34} is not a major binding site, which is surprising because Bi^{3+} has a high affinity for thiolate sulfur. This illustrates that the potential target sites for metals (in this case Bi^{3+}) in proteins depend not only on their presence but also on their accessibility. Bi^{3+} binds to transferrin in preference to albumin both in aqueous solution and in blood plasma.

Bismuth compounds have long been associated with medicine for the treatment of a variety of gastrointestinal disorders including diarrhea, constipation, gastritis, and ulcers (1–4). The effectiveness of bismuth has been attributed to its bactericidal action against the Gram-negative bacterium, *Helicobacter pylori*. There is also a growing interest in using compounds containing radioactive bismuth isotopes as targeted radiotherapeutic agents (5). However, the molecular basis for the mechanism of action of bismuth drugs is not well understood, including bismuth-induced toxicity, especially encephalopathy, which led to the withdrawal of bismuth drugs in France and Australia in the 1970s (1). The diagnosis of encephalopathy is generally defined by the detection of bismuth in blood, plasma or serum, the so-called "Hillemand safety level" (6, 7). Bismuth is primarily present in red blood cells, possibly binding to glutathione, with the remainder in serum or plasma (8–10). The speciation of bismuth in blood plasma, and in particular the nature of interactions of Bi^{3+} with plasma proteins, are in need of investigation.

Recently we have found that the binding of Bi^{3+} to human serum transferrin (hTF)[1] and recombinant N-lobe of transferrin is unexpectedly strong (11, 12). Transferrin is a single-chain glycoprotein (80 kDa) present in blood at a concentration of about 35 μM, and consists of two similar lobes, each of 40 kDa, connected by a short peptide. Its normal function in blood is to carry iron between sites of uptake, utilization, and storage (13–16). It contains two specific iron-binding sites per molecule, one in the N-terminal lobe and one in the C-terminal lobe. Iron binds as Fe^{3+} in a cleft formed by two domains in each lobe. Iron cannot bind strongly without concomitant binding of a synergistic anion. Since transferrin is only about 30% saturated with iron in normal serum (13, 17, 18), there is potential binding capacity for other metal ions that enter the blood. This has led to the idea that transferrin acts as a "delivery system" for therapeutic, diagnostic or toxic ions, including Ga^{3+}, Ru^{3+}, and Al^{3+} (19–21). Recently we have shown that Bi_2-hTF can block both membrane binding and cellular uptake of ^{59}Fe-hTF into BeWo placental cancer cells (22). It is therefore now important to establish whether Bi^{3+} binding to transferrin can occur under physiologically relevant conditions, especially in the presence of excess albumin and in blood plasma itself. We have shown previously that the order of lobe loading of hTF with metal ions can readily be determined via two-dimensional ^{1}H,^{13}C NMR studies of recombinant [ϵ-^{13}C]Met-hTF (23). It is known that the strength of binding to the two lobes is slightly different, and that Fe^{3+} is primarily situated in N-lobe in serum (14, 18).

Previous investigations of the interaction of Bi^{3+} with serum albumin has led to the suggestion that albumin may be the major target for Bi^{3+} in plasma (8), especially since albumin has a free thiolate group at Cys^{34}. Human serum albumin, the most abundant protein in blood at a concentration of about 40 mg ml^{-1} (about 0.63 mM, > 10 times that of transferrin), is a single-chain 66.5-kDa protein, which is largely α-helical, and consists of three structurally homologous domains (24). It is the major transport protein for unesterified fatty acids, but is also capable of binding an extraordinarily diverse range of metabolites, drugs, organic compounds, and metal ions, *e.g.* Ca^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+} (25, 26).

In the present work, the binding of a bismuth antiulcer drug

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[1] The abbreviations used are: hTF, human serum transferrin; [Bi(Hcit)], Bi(III) citrate; HSA, human serum albumin; HSQC, heteronuclear single-quantum coherence; hTF/2N, recombinant N-lobe of hTF; NTA, nitrilotriacetate; pH*, pH meter reading in D_2O; RBC, ranitidine bismuth citrate (an amorphous solid containing ranitidine, bismuth, and citrate in an approximate 1:1:1 molar ratio); ranitidine, *N,N*-dimethyl-5-(3-nitromethylene-7-thia-2,4-diazaoctyl)furan-2-methanamine; rHA, recombinant human albumin.

to human serum transferrin in aqueous solution in the presence of a large excess of albumin and to recombinant transferrin (N-lobe of transferrin and the mutant I132M of N-lobe of transferrin labeled with [ϵ-^{13}C]Met) in intact blood plasma has been monitored directly under physiologically relevant conditions using ^{1}H,^{13}C NMR spectroscopy. The introduction of Met^{132} into the N-lobe provides a convenient monitor for metal binding since this residue occupies a similar site within helix 5 of the N-lobe and forms part of the hydrophobic patch around Trp^{128} (Leu^{122}-Trp^{128}-Ile^{132}) as Met^{464} in the C-lobe (Val^{454}-Trp^{460}-Met^{464}). The interaction of bismuth with human albumin was also studied. Surprisingly, we found that Bi^{3+} still binds to the iron-binding sites of transferrin even in the presence of a large excess of albumin.

EXPERIMENTAL PROCEDURES

Materials—Recombinant N-lobe hTF/2N (residues 1–337) was expressed in baby hamster kidney cells using a pNUT plasmid with L-[ϵ-^{13}C]methionine in the growth medium, and purified as described previously (27, 28). A gene for the mutant I132M protein was created by site-directed mutagenesis using previously published methods (27, 29). Iron was removed from proteins by treatment with a metal-removal buffer containing 1 mM NTA, 1 mM EDTA, and 0.5 M sodium acetate, pH 4.9, using ultrafiltration Centricon 10 ultrafilters (Amicon). Human serum albumin (HSA) was purchased from Sigma as essentially globulin-free and fatty acid-free and was purified via ultrafiltration (Centricon 10) using 0.1 M KCl and washing 3 times (each 1 h). It was then lyophilized. Recombinant human albumin (rHA) was supplied by Delta Biotechnology Ltd. (batches GA 950202 and R970103). Samples of rHA were dialyzed against 100 mM ammonium bicarbonate, pH 7.9, and freeze-dried. Ranitidine bismuth citrate (RBC) and bismuth citrate [Bi(Hcit)] were provided by GlaxoWellcome plc. $NaHCO_3$, KCl, 5,5′-dithiobis(2-nitrobenzoic acid), and other chemicals were purchased either from Aldrich or Sigma with the highest quality and used as received. Crystalline [Bi(NTA)] was synthesized according to a literature procedure (30), and had a satisfactory elemental analysis.

A 50 mM stock solution of $[Bi(cit)]^-$ was prepared by addition the minimum amount of ammonia solution to a suspension of [Bi(Hcit)] until the solution became clear. The final pH of this solution was about 7, and it was then diluted before use. A solution of $Fe(NTA)_2$ was prepared from an iron atomic absorption standard (1000 ppm in 1% HNO_3, Aldrich) and 2 mol eq of H_3NTA (Aldrich), followed by pH adjustment to between 6.0 and 7.0. This was lyophilized and redissolved in D_2O before use. A [Bi(NTA)] solution was prepared by dissolving a known amount of [Bi(NTA)] in D_2O.

Preparation of NMR Samples—I132M [ϵ-^{13}C]Met hTF/2N (0.15 mM) and mixtures with either rHA (1.8 mM) or HSA (1.8 mM) were prepared in D_2O containing 0.1 M KCl. Prior to Bi^{3+} or Fe^{3+} titrations, an aliquot of concentrated $NaHCO_3$ (0.25 M) was added to the samples to give a final concentration of 10 mM, and pH* values were adjusted using NaOD or DCl. For the intact blood plasma experiments, blood from a male healthy volunteer was collected by venipuncture into lithium-heparinized vacutainers, and the plasma was separated by centrifugation at 6000 rmp for 20 min at 277 K, and stored frozen until used for NMR measurements. I132M hTF/2N was added to 1.2 ml of intact blood plasma to give a hTF/2N concentration of 50 μM and the concentration of this sample was doubled by freeze-drying and reconstitution in 0.6 ml of D_2O, followed by addition of concentrated $NaHCO_3$ to a final concentration of 20 mM. The pH* was then measured. After addition of Bi^{3+}, samples were left to equilibrate for at least 30 min at 310 K. All experiments on reconstituted blood plasma were carried out at pH* 7.8 since it was possible to maintain this as a stable pH* value during the course of the long NMR data accumulations. If the initial pH* value was lower, it tended to drift upwards during the experiment. The ^{1}H,^{13}C chemical shifts of the [ϵ-^{13}C]Met resonances of transferrin were insensitive to pH over the range of 7–8.8, and NMR experiments on Bi^{3+} loading of transferrin with [Bi(NTA)] and ranitidine bismuth citrate gave the same results at pH* 7.4 and pH* 7.8.

NMR Spectroscopy—NMR spectra were recorded on a Bruker DMX500 spectrometer at 310 K. For one-dimensional ^{1}H NMR spectra, 400 to 1200 transients were acquired with 6-μs (50°) pulses and 16,384 data points during the 2-s pulse delay and the water resonance was suppressed via presaturation. For two-dimensional ^{1}H,^{13}C heteronuclear single quantum coherence spectra (HSQC) experiments, the sequence was optimized for ^{1}J (^{1}H-^{13}C) = 140 Hz, and 16 to 32 transients were acquired using 2,048 data points in the f_2 dimension (^{1}H), 32 to 64 increments in the f_1 dimension, ^{13}C frequency width of 3 kHz, and relaxation delay of 1.6 s. The ^{13}C spins were decoupled using the GARP sequence (31). After zero-filling to 4,096 × 1,024 data points, unshifted Gaussian functions were used for processing in both dimensions. Water suppression was achieved by a combination of presaturation and pulsed-field gradients. One-dimensional HSQC NMR spectra (or ^{13}C-edited ^{1}H NMR spectra) were recorded using the first increment of the two-dimensional HSQC sequence. Resolution enhancement was achieved using a combination of exponential (1.5 to 10 Hz line-broadening) and unshifted sine-bell functions (32). Peaks were referenced to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 via the external ϵ-CH_3 peak of L-methionine (15.14 ppm) for ^{13}C and via formate (8.465 ppm, a minor impurity always present in the protein) for ^{1}H.

Determination of Bi—HSA (concentration about 0.1 mM) was incubated with different mole ratios of RBC in 0.1 M Tris-HCl buffer, pH 7.4, overnight at 310 K. Unbound RBC was separated via gel filtration using a Superose 12 column and a FPLC system (Amersham Pharmacia Biotech). The concentration of albumin samples was about 6 mg/ml and 500 μl of the protein solution was loaded onto the column. Elution conditions were 0.1 M Tris-HCl, pH 7.4, and flow rate 0.5 ml/min. The fractions eluting from 10 to 14 ml were collected and the bismuth content was determined using a CETAC Microneb 2000 direct injection nebulizer (CETAC Technologies, Omaha, NE) coupled with a VG PlasmaQuad PQ2 ICP-MS instrument (VG Elemental, Winsford, Cheshire, UK). Details of the optimization procedure for the DIN-ICP-MS system and measurement conditions for bismuth have been described previously (33).

Determination of Albumin Thiol Content—The free SH content of albumin was determined after incubation with RBC or $[Bi(cit)]^-$ using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid). HSA or rHA were incubated with different mole ratios of RBC or $[Bi(cit)]^-$ overnight (>12 h) in 0.1 M Tris-HCl buffer, 310 K, pH 7.4, and unbound bismuth was removed by ultrafiltration using Centricon 10 ultrafilters. The concentrations of HSA and rHA were determined using ϵ_{279} values of 35,300 and 37,100 M^{-1} cm^{-1}, respectively (25, 34), and the amount of nitrobenzoic acid thiol generated was calculated using ϵ_{412} = 13,600 M^{-1} cm^{-1} (35).

RESULTS

NMR Studies of Bi^{3+} Binding to I132M hTF/2N and Comparison with Fe^{3+}

One-dimensional ^{1}H NMR—It has been shown previously that ^{1}H NMR spectra of human serum transferrin are complicated by the overlap of the very large number of resonances present and by their broadening due to the slow tumbling of this 80-kDa protein (36). However, the high-field region of the spectrum of the N-lobe is relatively well resolved. High field-shifted resonances have been assigned to protons from residues around Trp^{128}, *i.e.* Leu^{122}, and Ile^{132} (37, 38). The mutation of Ile^{132} to Met should lead to the disappearance of the resonance at −0.603 ppm, which has been previously assigned as γCH_2 of Ile^{132}. Indeed, this was found to be the case (Fig. 1). Other changes were also observed in the spectrum of the mutant in comparison to that of wild-type hTF/2N. For example, the resonance at −0.170 ppm for hTF/2N disappeared, and the peak for δCH_3 of Leu^{122} (−0.339 ppm in hTF/2N) shifted to −0.324 ppm. Addition of 0.5 mol eq of Bi^{3+} (as [Bi(NTA)]) caused new peaks to appear at −0.160 ppm (C′), and −0.255 ppm (B′), and further addition of Bi^{3+} (total 1.0 mol eq) increased the intensity of both C′ and B′ but decreased that of peak B significantly (Fig. 1*A*).

For comparison, titrations of Fe^{3+} (added as $Fe(NTA)_2$) with the mutant protein were also performed under similar conditions (pH* 7.8, 310 K and 10 mM bicarbonate) and the results are shown in Fig. 1*B*. Upon addition of 0.5 mol eq of Fe^{3+}, the resonance at −0.324 ppm decreased in intensity, and almost completely disappeared with 0.9 mol eq Fe^{3+} present. Broad new peaks at −0.004 and −0.392 ppm appeared and increased in intensity. It is reasonable to assume from these titration studies that the resonance at −0.324 ppm consists of two overlapped peaks, one of which (peak B) can be assigned to the

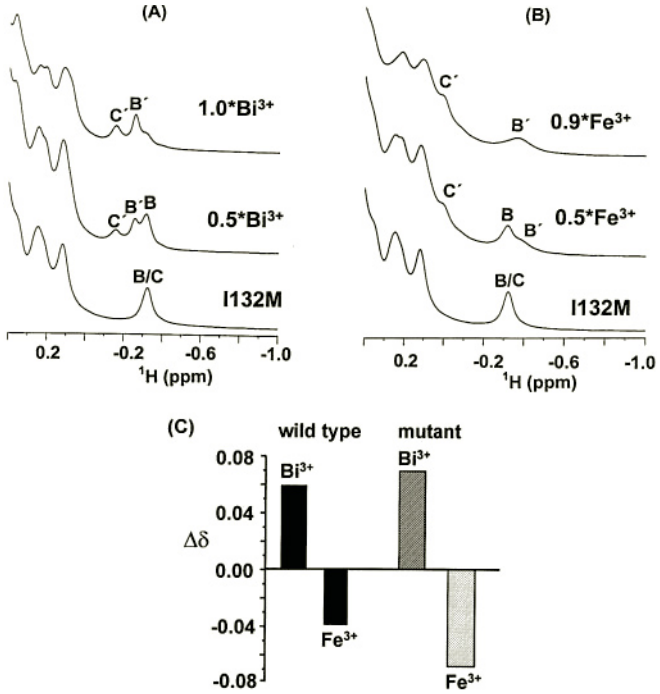

FIG. 1. **The high-field region of the ^{1}H NMR spectrum of apo-I132M hTF/2N and its Bi^{3+} and Fe^{3+} complexes in the presence of 10 mM bicarbonate 0.1 M KCl, pH* 7.8.** *A,* apo-I132M hTF/2N (*bottom*), and after addition of 0.5 (*middle*), and 1.0 mol eq of [Bi(NTA)]; *B,* apo-I132M hTF/2N (*bottom*), and after addition of 0.5 (*middle*) and 0.9 mol eq of $[Fe(NTA)_2]$; *C,* stack plot showing the changes in shifts of the methyl protons of Leu^{122} for wild-type hTF/2N and I132M mutant after metal binding.

δCH_3 of Leu^{122} as judged from the change in pattern of this peak on titration of both wild-type and mutant proteins with metal ions. The other peak (C) cannot be assigned. Two-dimensional total correlation spectroscopy and NOESY experiments support these assignments (data not shown). Peak B has identical associated NOESY cross-peaks for both wild-type and mutant hTF/2N, which suggests that peak B belongs to the δCH_3 of Leu^{122}. Similar cross-peak patterns were observed for B and B′ in the NOESY spectrum, which indicates that peak B′ for the metal-bound protein is the analogue of peak B. A comparison of the changes in the chemical shifts of the δCH_3 peak of Leu^{122} after binding of hTF/2N and I132M-hTF/2N to Bi^{3+} and Fe^{3+} is shown in Fig. 1*C*.

Two-dimensional ^{1}H,^{13}C HSQC NMR—The two-dimensional ^{1}H,^{13}C HSQC NMR spectrum of I132M hTF/2N and after addition of 0.5 mol eq of Bi^{3+} or Fe^{3+}, in the presence of 10 mM bicarbonate, are compared in Fig. 2. As expected, six cross-peaks were observed for the apo-protein, five of which have similar chemical shifts as those observed for the wild-type protein. These have been assigned previously on the basis of single-site mutations combined with other considerations (39, 40). Therefore the sixth peak, at 1.51/17.98 ppm, can be assigned to Met^{132}. On addition of 0.5 mol eq of Bi^{3+} (added as either RBC or as [Bi(NTA)]), a notable decrease in intensity of the latter peak occurred and a new peak appeared at 1.45/17.93 ppm, which can be associated with the bound form of the protein. Other new peaks, which can be assigned to Met^{109} and Met^{309}, also appeared and shifted slightly in both ^{1}H and ^{13}C dimensions (Table I). Further addition of Bi^{3+} (1.0 mol eq) caused the disappearance of the peaks at 1.51/17.98, 1.94/16.15, and 2.15/16.16 ppm and led to a further increase in the intensity of the new peaks. These changes were observed more clearly in high-resolution one-dimensional ^{13}C-edited ^{1}H spectra (see below, Fig. 3).

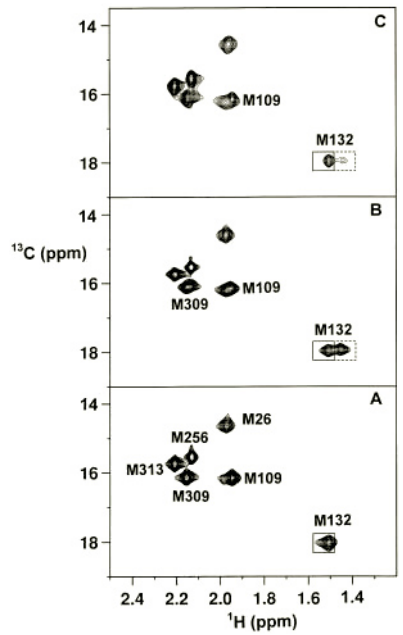

FIG. 2. **Two-dimensional ^{1}H,^{13}C HSQC NMR spectra of [ε-^{13}C]Met I132M hTF/2N in 10 mM bicarbonate, 0.1 M KCl at pH* 7.8.** *A,* apo-hTF/2N; *B,* after addition of 0.5 mol eq of ranitidine bismuth citrate; and *C*, apo-hTF/2N after addition of 0.5 mol eq of Fe^{3+} (added as $[Fe(NTA)_2]$).

TABLE I
^{1}H and ^{13}C NMR chemical shifts of the [ε-^{13}C]Met resonances of apo-I132M hTf/2N (in 10 mM HCO_3^-) and its Bi^{3+} complex at 310 K

Residue	Apo-form δ/ppm, δ (^{1}H/^{13}C)	Bi-bound	
		δ (^{1}H/^{13}C)	Δδ (^{1}H/^{13}C)
Met^{26}	1.97/14.65	1.97/14.60	0.00/−0.05
Met^{109}	1.94/16.15	1.97/16.22	0.03/0.07
Met^{132}	1.51/17.98	1.45/17.93	−0.06/−0.05
Met^{256}	2.13/15.54	2.13/15.54	0.00/0.00
Met^{309}	2.15/16.16	2.14/16.10	−0.01/−0.06
Met^{313}	2.21/15.76	2.20/15.75	−0.01/−0.01

Addition of 0.5 mol eq of Fe^{3+} (added as $[Fe(NTA)]_2$) to I132M hTF/2N caused similar changes in the two-dimensional HSQC spectrum as for 0.5 mol eq Bi^{3+}, except that the peak for Met^{132} for the bound form was significantly broadened. The resonance for Met^{132} in the apo-protein disappeared after addition of 1.0 mol eq of Fe^{3+}. The changes to the shifts of the other Met resonances were identical to those observed on addition of 1.0 mol eq of Bi^{3+}.

Binding of Bi^{3+} to I132M hTF/2N in the Presence of Excess of Albumin—The ^{1}H and ^{13}C NMR chemical shift changes induced by metal ions (*e.g.* Fe^{3+} and Bi^{3+}) provide convenient probes for investigation of Bi^{3+} translocation between transferrin and proteins such as albumin. These experiments were performed using low concentrations of I132M hTF/2N (150 μM) in the presence of 12 mol eq of HSA or rHA (1.8 mM), pH* 7.8, 10 mM bicarbonate, 310 K. We choose an [albumin]/[transferrin] ratio of 12:1 to mimic biological conditions. The concentration of albumin in blood plasma (about 0.63 mM) is about 18 times higher than that of transferrin (about 35 μM), but hTF is only about 30% saturated with Fe^{3+}. The ^{1}H,^{13}C two-dimensional HSQC NMR spectrum of this protein mixture shows sharp resonances from the six labeled Met residues of transferrin and broadened (natural abundance) resonances from albumin. The Met^{132} peak was overlapped with peaks from albumin (data not shown). Since I132M hTF/2N is present at low concentration, one-dimensional ^{13}C-edited ^{1}H NMR spectra were recorded over a period of 30 min each. Fig. 3 shows the

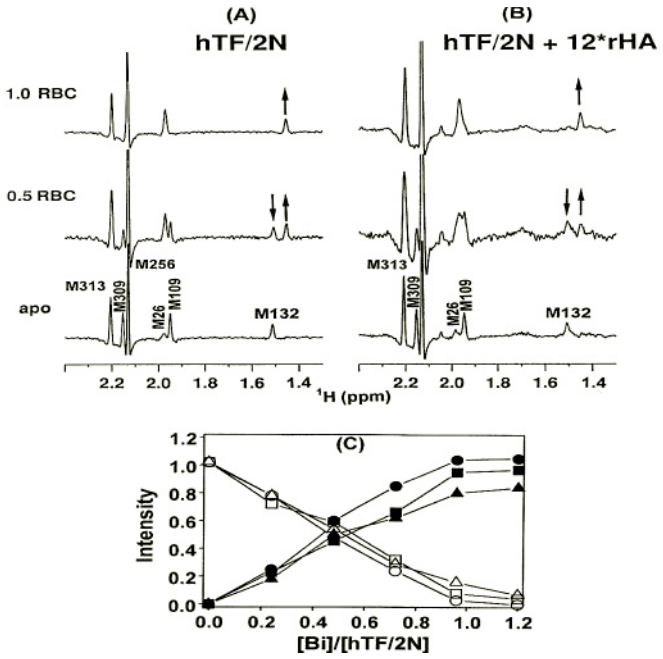

FIG. 3. **Effect of the antiulcer compound ranitidine bismuth citrate on the one-dimensional ^{13}C-edited ^{1}H NMR spectrum of apo-I132M hTF/2N.** *A,* in the absence; and *B,* in the presence of 12 mol eq of recombinant serum albumin. *C,* variation in intensity of the ^{13}C-edited ^{1}H NMR peak for Met^{132} in [ϵ-^{13}C]Met-I132M hTF/2N with increase in the Bi^{3+}/protein ratio. *Open symbols,* apo-hTF/2N; *filled symbols,* Bi-hTF/2N; *circle,* hTF/2N; *square,* hTF/2N + 12 mol eq of rHA; *triangle,* hTF/2N + 12 mol eq of HSA.

^{13}C-edited ^{1}H NMR spectrum of I132M hTF/2N in the absence and presence of recombinant albumin (rHA). Broad resonances at 1.70 and from 1.45 to 1.10 ppm from albumin were filtered out with resolution enhancement using a combination of unshifted sine-bell and exponential functions prior to Fourier transformation.

The antiulcer compound, ranitidine bismuth citrate, was added in 0.25 mol eq steps to I132M hTF/2N in the presence of 12 mol eq of rHA, 10 mM bicarbonate. With increase in Bi^{3+} concentration, a new peak at 1.45 ppm appeared. This can be assigned to Met^{132} in Bi-I132M hTF/2N, and it gradually increased in intensity, reaching a maximum after 1 mol eq of Bi^{3+} had been added. The peak for Met^{132} in the apo-protein simultaneously decreased in intensity and finally disappeared (Fig. 3). The change in the peak for Met^{309} (2.15 ppm) was observable more clearly in one-dimensional ^{1}H{^{13}C} spectra: it gradually decreased in intensity with the addition of Bi^{3+} and finally disappeared. The resonance for Met^{309} is probably that at 2.14 ppm, which was overlapped with Met^{256}, as can be judged from the increased intensity in the two-dimensional HSQC spectrum (data not shown). The peak for Met^{109} also appears to shift to low field by 0.03 ppm. The same chemical shift changes were observed after addition of Bi^{3+} to I132M hTF/2N in the presence of 12 mol eq of HSA. The lack of effect of albumin on Bi^{3+} binding to this transferrin N-lobe is clearly illustrated in Fig. 3*C*, which shows the integrated intensity of the peak for Met^{132} after addition of various amounts of Bi^{3+} in the absence and presence of albumin.

Since the concentration of albumin present in the sample is much higher than that of transferrin, the ^{1}H NMR spectrum is dominated by peaks from albumin. The aromatic region of spectra of these mixed protein solutions (hTF/2N + 12rHA or + 12HSA) was almost identical with and without addition of Bi^{3+}, particularly the resonance at 7.632 ppm, which has been

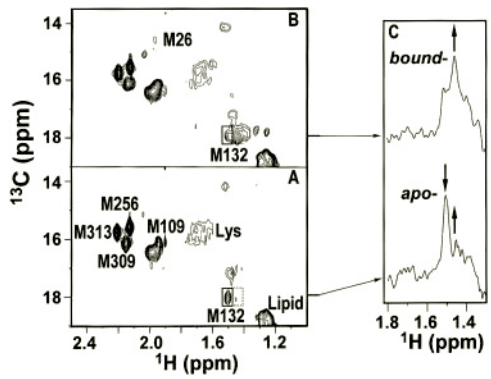

FIG. 4. **Two-dimensional ^{1}H,^{13}C HSQC NMR spectra of blood plasma containing 100 μM [ϵ-^{13}C]Met I132M apo-hTF/2N.** *A,* before; *B,* after addition of Bi^{3+} (ranitidine bismuth citrate); and *C,* slices through two-dimensional ^{1}H,^{13}C HSQC spectra in the ^{1}H dimension (corresponding to the ^{13}C signal of $-S\underline{C}H_3$ of Met^{132}). The shift of the peak for Met^{132}, which is in the hydrophobic patch of helix 5, is similar to that observed after direct addition of Bi^{3+} to I132M apo-hTF/2N (Fig. 2).

previously assigned (41, 42) to His^{3} of albumin (data not shown).

Uptake of Bi^{3+} by Transferrin in Plasma

The concentration of transferrin in human plasma is about 35 μM. It is only about 30% saturated with iron (18) and therefore has about 50 μM capacity for binding to other metal ions. To determine if transferrin is a target for bismuth, isotopically labeled [ϵ-^{13}C]Met I132M hTF/2N (50 μM) was directly added to human plasma. The whole plasma concentration (including the added transferrin) was lyophilized, and the sample was redissolved in half-volume of the original plasma solution. This gave an I132M hTF/2N concentration of 100 μM.

Even with resolution enhancement, the peak for Met^{132} was still overlapped with other peaks in the ^{13}C-edited ^{1}H NMR spectrum (data not shown). Therefore only the two-dimensional HSQC method was used. The two-dimensional HSQC spectrum of this solution containing 100 mM KCl and 20 mM bicarbonate is shown in Fig. 4. Surprisingly, the peak for Met^{109} became severely broadened but the rest of the Met cross-peaks from transferrin were clearly observed. Many other cross-peaks are present but are difficult to assign, partly due to the limited frequency width used (12 ppm in ^{13}C dimension). The peaks at about 1.46/17.2 ppm, and 1.67/15.8 ppm (folded in ^{13}C dimension) can be assigned to Ala and Lys residues, respectively, of albumin, and the peaks at about 1.24/19.2 ppm to lipids in plasma. After addition of 0.5 mol eq of RBC (relative to the available transferrin-binding sites), the peak for Met^{132} (1.51/17.98 ppm) in apo-hTF/2N decreased in intensity and the peak at 1.45/17.93 ppm for Bi^{3+}-I132M hTF/2N increased in intensity. Similarly, the peak for Met^{109} (1.94/16.15 ppm) disappeared and a new peak (bound form) appeared at slightly lower field, and that for Met^{309} (2.15/16.16 ppm) shifted to high field. The cross-peak for Met^{132} in the apo-protein almost disappeared and the analogous peak for the bound-form further increased in intensity (Fig. 4). After addition of 1.0 mol eq of RBC, this behavior was similar to that observed for I132M hTF/2N with and without 12 mol eq of serum albumin or recombinant albumin under same conditions. Interestingly, with Bi^{3+} bound to the protein, the peak for Met^{26} became sharper and observable.

A second experiment was carried out with blood plasma containing twice the concentration of I132M (200 μM), but the behavior of the Met two-dimensional cross-peaks on titration with RBC was similar. The normal one-dimensional ^{1}H NMR

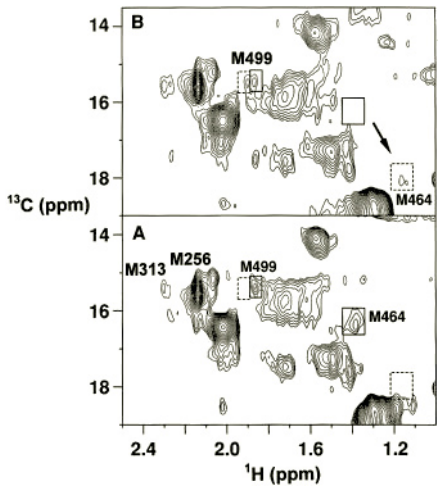

FIG. 5. **Two-dimensional ^{1}H,^{13}C HSQC NMR spectra of (*A*) [ε-^{13}C]Met apo-hTF (35 μM) in blood plasma at 310 K, and (*B*) after addition of 1 mol eq Bi^{3+} as ranitidine bismuth citrate.** The *solid boxes* indicate initial peaks and *dotted boxes* show new peaks (Bi^{3+} bound form).

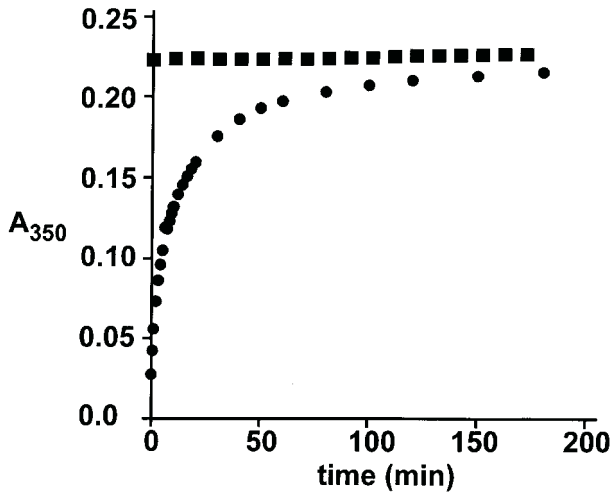

FIG. 6. **Time dependence of the reaction of glutathione (40 mol eq) with Bi^{3+} albumin (*circle*) or Bi^{3+} citrate ($[Bi(cit)]^{-}$, *square*) as measured by the absorbance at 350 nm (due to $Bi(SG)_3$).**

spectrum of plasma in the His region was identical in the absence and presence of Bi^{3+}, especially the peak for His^{3} of albumin (7.632 ppm), which has previously been used as an indicator for drug binding at Cys^{34} (41).

The intact protein as [ε-^{13}C]Met-transferrin was also added directly to 1.2 ml of human plasma to give a concentration of 35 μM, and the concentration of the sample was doubled by freeze-drying and dissolving in 0.6 ml of D_2O in the presence of 20 mM sodium bicarbonate. Most of the Met resonances were observable in the two-dimensional HSQC spectrum except those for Met^{26}, Met^{309}, and Met^{389} (Fig. 5). Other peaks from plasma were also observed from groups present at relatively high concentrations such as Lys (from albumin) and lipids. The notable change was for Met^{464} in the C-lobe from its apo-position (1.38/16.30 ppm for ^{1}H/^{13}C) to Bi^{3+} bound form (1.18/18.2 ppm for ^{1}H/^{13}C) after addition of 1 mol eq of ranitidine bismuth citrate. A similar change also occurred for Met^{499} in the C-lobe.

Interactions of Bismuth Complexes with Albumin

Effect of Bismuth on the Free Thiol Content of Albumin—The free thiol of albumin at Cys^{34} is a potentially strong binding site for Bi^{3+}. The thiol contents of human serum albumin and recombinant human albumin were determined before and after reaction with bismuth citrate (either RBC or $[Bi(cit)]^{-}$) by the 5,5′-dithiobis(2-nitrobenzoic acid) method. The rHA (recombinant) sample contained 0.77 ± 0.01 mol of thiol/mol of protein, while thiol content of (isolated) HSA was significantly lower, only 0.29 ± 0.01 mol/mol HSA. After reaction with various amounts of bismuth citrate in 0.1 M Tris-HCl buffer at pH 7.4 for 12 h, the SH contents decreased by less than 12%, from 0.77 to 0.68 for rHA and from 0.29 to 0.26 for HSA, respectively. This suggests that little Bi^{3+} binds to Cys^{34} of albumin.

Determination of Amount of Bismuth Bound to Human Serum Albumin—Various mole ratios of ranitidine bismuth citrate were reacted with albumin in 0.1 M Tris-HCl buffer at pH 7.4 and equilibrated overnight at 310 K. Albumin-bound bismuth was then separated from free bismuth by gel filtration chromatography. The Bi^{3+} content of the albumin fractions was measured by DIN-ICP-MS (data not shown). The amount of Bi^{3+} bound to albumin increased almost linearly with increase in added RBC and did not reach saturation even with 25 mol eq of RBC present. The gel filtration chromatograms of control albumin and its complex with bismuth were very similar both in terms of peak intensity and retention time (data not shown) suggesting that bismuth does not cause aggregation of the protein. When 40 mol eq of glutathione (relative to the measured Bi^{3+}) was added to the albumin fraction, a new broad band centred at about 350 nm gradually increased in intensity in a multiphase process, and reached a maximum intensity over a period of 3 h (Fig. 6). This is in contrast to the reaction of bismuth citrate alone with 40 mol eq of glutathione under similar conditions which was complete within minutes. The band at 350 nm is a typical Bi-S absorbance indicating formation of $[Bi(SG)_3]$ (9).

DISCUSSION

Bismuth compounds are widely used as antiulcer drugs and recently we have shown (22) that bismuth transferrin, Bi_2-hTF, exhibits marked dose-dependent effects on membrane binding and cell uptake of ^{59}Fe-hTF by placental BeWo cells. This suggested that bismuth transferrin is recognized by the transferrin receptor. The present study was undertaken to determine whether Bi^{3+} can bind to transferrin under physiological conditions, especially in the presence of excess albumin, and in blood plasma itself. Previously we have shown that NMR can be used to monitor the uptake of metals into the individual lobes of transferrin (23). The ^{1}H,^{13}C NMR cross-peak for Met^{464} of human transferrin is a sensitive indicator of metal binding to the C-lobe since significant chemical shift changes are induced in both ^{1}H and ^{13}C dimensions. In the N-lobe of intact hTF, however, there is lack of this kind of sensitive indicator. Met^{464} is situated in the hydrophobic patch (Val^{454}-Trp^{460}-Met^{464}) of helix 5 in the C-lobe (Fig. 7), which backs onto the metal-binding site and H-bonds to the synergistic anion (43, 44). In the N-lobe there is a similar hydrophobic patch in helix 5 near the metal-binding site, consisting of Leu^{122}, Trp^{128}, and Ile^{132} (Fig. 7) (45). The analogue of Met^{464} is Ile^{132} in human serum transferrin, but is Met^{132} in cow and pig transferrin (46), which suggests that I132M is a structurally conservative substitution. To provide a possible sensitive indicator for metal ion binding in the N-lobe of human serum transferrin and to investigate the similarity between the two lobes of transferrin, Ile^{132} was mutated to Met using site-directed mutagenesis. It is easy to produce N-lobe protein in this way in the quantities required for NMR. In contrast, recombinant C-lobe is difficult to prepare, but the N-lobe and C-lobe metal binding constants are usually close (47).

The ^{1}H NMR spectrum of I132M hTF/2N was similar to that of wild-type hTF/2N in the both high-field and His C2H regions, except for the disappearance of the peak for the γCH_2 of

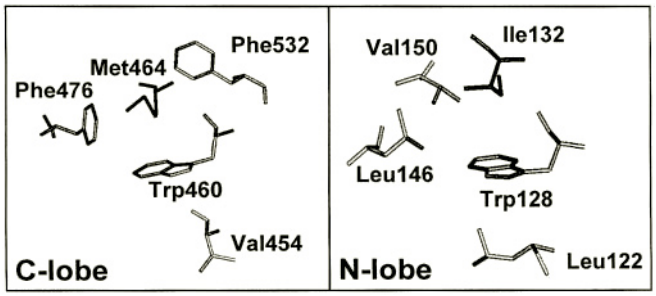

FIG. 7. **Comparison of the hydrophobic patches around Trp^{128} in the N-lobe and Trp^{460} in the C-lobe of transferrin showing the positioning of Ile^{132} and Met^{464}, and Leu^{122} and Val^{454} above and below, respectively, the indole rings of the Trp residues (based on PDB entry 1A8E (45)).**

Ile^{132} at −0.603 ppm. Both Bi^{3+} and Fe^{3+} induce similar chemical shift changes for the high field-shifted peak for δCH_3 of Leu^{122} in the mutant and wild-type hTF/2N (Fig. 1). This suggests that the overall structure of the mutant is similar to that of wild-type hTF/2N. This was also confirmed by molecular modeling, which showed that the protein backbone fold of the mutant is almost identical to that of the wild-type protein. Six of the 10 lowest energy structures placed the side chain of Met^{132} above Trp^{128} (data not shown), a situation which would give rise to a ring current shift for the ϵ-CH_3 of Met^{132}.

In the two-dimensional ^1H,^{13}C NMR spectrum of apo-I132M hTF/2N, the ϵ-$^{13}CH_3$ resonance of Met^{132} exhibits a significant ^1H NMR high-field shift compared with the rest of the Met peaks, as does the analogous cross-peak for Met^{464} in the C-lobe. Only small changes in shifts of the Met^{132} resonance ($\Delta\delta$ −0.06/−0.05 ppm for ^1H and ^{13}C, respectively, Table I) occurred when Bi^{3+} or Fe^{3+} binds to the mutant I132M hTF/2N, in contrast to the large shifts for Met^{464} ($\Delta\delta$ −0.20/1.90 ppm for ^1H and ^{13}C, respectively) suggesting that the structural changes in helix 5 on loading the protein with metal ions are slightly different for the N- and C-lobes. X-ray crystallographic studies have shown that when metals bind and domain closure occurs, helix 5 pivots on helix 11 and that a domain movement of about 54 ° occurs in the N-lobe but only about 15° rotation in the C-lobe (48, 49).

Our studies suggest, for the first time, that transferrin should be considered as a potential mediator for bismuth transport in blood plasma. Previously, it has been assumed (8) that albumin, the most abundant protein in blood serum with a free thiol group at Cys^{34}, is a target site for bismuth drugs, since Bi^{3+} is known to have a high affinity for thiolate sulfur. Glutathione, a thiolate sulfur-containing peptide (GSH), for example, can readily displace Bi^{3+} from its complexes with citrate and EDTA at biological pH values (9). Recent reports (8) have shown that only 2% of albumin molecules bind to Bi^{3+} if binding is assumed to occur at the free thiol group of Cys^{34} (pK_a about 5 (50)). In this work we have demonstrated that binding of Bi^{3+} to albumin is nonspecific; even 25 mol eq of Bi^{3+} did not saturate albumin, and Cys^{34} is not blocked by Bi^{3+} binding. Previous ^1H NMR studies of albumin have shown that the imidazole CH resonances of His^3 are sensitive to the oxidation of Cys^{34} and to the formation of adducts with gold antiarthritic drugs (41) probably because such reactions lead to movement of the side chain of Cys^{34} which is communicated to His^3 via intervening helices. The His regions of ^1H NMR spectra of albumin in the presence of I132M hTF/2N or of blood plasma in the presence of intact hTF were found to be almost identical after addition of bismuth compounds (data not shown) which provide further evidence that Cys^{34} is not a major binding site for Bi^{3+}.

We have successfully used two-dimensional HSQC NMR spectroscopy to probe changes of Met resonances of transferrin in solution in which the concentration of albumin is 10 times higher. The observation of similar changes in the chemical shifts of the Met resides of I132M hTF/2N on binding Bi^{3+} in the presence or absence of a large excess of albumin, and even in blood plasma, suggests that similar conformational changes are induced by Bi^{3+} under these conditions. Such structural changes could be important for recognition by the transferrin receptor. Bi^{3+} was also observed to bind to intact transferrin in the presence of a large excess (12 mol eq) of serum albumin or recombinant albumin and a similar behavior was observed in blood plasma. Our findings may have implications for the mechanism of neurotoxicity of bismuth drugs (encephalopathy). For a long time it has not been clear how bismuth is transferred to the brain. It is generally accepted that the diagnosis of bismuth encephalopathy can be confirmed by the detection of high Bi^{3+} levels in whole blood, serum, or plasma, the so-called Hillemand safety level (6). It is likely that once bismuth has entered into blood it is transported by transferrin, in a similar manner to Al^{3+}. Al^{3+} deposition in the brain is known to cause dialysis encephalopathy and this neurotoxicity is related to transferrin transportation and transferrin receptor recognition in the brain (51).

Selective labeling of the protein in combination with inverse NMR detection is a powerful method for probing the structure and dynamics of high molecular mass proteins, and provides an approach for investigating the translocation of metallo-drugs (and other drugs) between proteins and enzymes at concentrations of biologically relevance without separation, and can also be applied to protein-ligand (in this case for drug screening) (52) and protein-protein interactions.

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